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NOT JUST PROTEIN FACTORIES: ROLE OF RIBOSOME BIOGENESIS AS AN ARCHITECT OF EPITHELIAL-MESENCHYMAL TRANSITION AND BREAST CANCER PROGRESSION

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Not Just Protein Factories: Role of Ribosome Biogenesis as an Architect of Epithelial-Mesenchymal Transition and Breast Cancer Progression

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Dedicated to all the souls touched by cancer

Whatever has happened, has happened well
Whatever is happening, is happening well
Whatever is due for the future, will happen well
What did you lose? Why do you worry?
What did you bring with you, that you think you have lost?
What did you create, that you think you have destroyed?
Whatever you have, you received here
Whatever you gave, you gave it here
Whatever is yours today will be someone else's tomorrow
Another day it will be somebody else's
This is the World’s ordinance and essence of My creation

-The Bhagavad Gita

ABSTRACT

RNA polymerase I (Pol I)-mediated transcription of ribosomal DNA (rDNA) is considered to be the rate-limiting step in ribosome biogenesis and is a well-known hallmark of cell growth and proliferation. The process of synthesizing new ribosomes is executed by the coordination of multiple complex processes in the nucleolus. The initial step of transcribing 47S ribosomal RNA (rRNA) transcript by the Pol I complex is followed by its processing into 28S, 18S and 5.8S rRNAs. These transcripts, together with 5S rRNA transcribed by Pol III and auxiliary proteins transcribed by Pol II, proceed to form a mature ribosome after being exported into the cytoplasm. Regulation of ribosome biogenesis occurs in a cell cycle dependent manner, and actively transcribing nucleolar organizing regions (NORS) indicating active rDNA transcription have been associated with tumor proliferation and poor prognosis in cancer patients. Numerous oncogenic and tumor suppressive pathways modulate tumor growth through rDNA transcription. We have previously shown that the tumor suppressive effects of Wnt5a is mediated though suppression of rDNA transcription by recruitment of Dishevelled 1 (DVL1) to the nucleolus and the rDNA gene cassette. In this thesis, we show that *de novo* ribosome biogenesis is essential for the epithelial-to-mesenchymal transition (EMT), which is indispensable for embryonic development and for the acquisition of migratory phenotype during cancer progression. The induced *de novo* rRNA synthesis occurring in the absence of cell proliferation is mediated by increased recruitment of Pol I complex components and EMT transcription factor Snail1 to the rDNA gene cassette. This is accompanied by the opening of the otherwise silenced rDNA operons by the release of TTF-I interacting protein 5 (TIP5), a major component of the repressive nuclear chromatin remodeling NoRC complex, from the rDNA. Pharmacological inhibition of rRNA synthesis by the small molecule CX-5461 reduced the invasive capacity of cells *in vitro,* which correlated with a decrease in mesenchymal proteins, together confirming an important role of *de novo* ribosome biogenesis in EMT. In accordance with previous literature that have shown association of the mTORC2 complex with ribosomes, expression of Rictor, a mTORC2 complex component, was found to be induced in the nucleolus during EMT. This association of Rictor was observed to be rRNA dependent. Furthermore, inhibition of ribosome biogenesis significantly reduced the nucleolar expression of Rictor. Mouse models of metastatic breast cancer showed reduced tumor volume upon treatment with CX-5461 and a significant reduction in lung metastasis was observed. Interestingly, CX-5461 treated primary tumors were also more differentiated, as they had increased expression of cytokeratin 8/18, and were also Estrogen Receptor-alpha (ERα) positive and Rictor-negative, which altogether correlates with a less aggressive phenotype in

the MMTV-PyMT mouse tumor model. Further investigation into the driving mechanism of EMT by *de novo* ribosome biogenesis revealed pervasive changes in the translational control of gene expression program during EMT. This translational control during EMT was affected by inhibition of *de novo* rRNA synthesis by the Pol I assembly inhibitor, CX-5461. Though the transcriptional profiles remained the same, about 1478 genes were differentially expressed in the ribosome protected fragments during EMT. CX-5461 treatment blocked the upregulation of 185 and the downregulation of 179 translationally controlled genes. The expression of the translationally controlled genes post-TGFβ stimulation significantly overlapped with the translationally controlled genes affected by CX-5461. Interestingly, a translational reprogramming of the mTORC1 signaling cascade was observed during EMT, revealed by the downregulation of transcripts with short UTRs. This reprogramming was diminished by the inhibition of Pol I mediated rDNA transcription. These findings collectively provide compelling evidence that the EMT-associated ribosome biogenesis program, and by extension the ribosomes generated by this process, fuel the pro-migratory, pro-invasive gene expression program underpinning EMT and thus the mesenchymal phenotype. They also demonstrate compelling evidence that rRNA biogenesis plays a unique and targetable role in metastatic breast cancer development, progression and metastasis.

LIST OF SCIENTIFIC PAPERS

I. **Ribosome biogenesis during cell cycle arrest fuels EMT in development and disease**
*Varsha Prakash**, Brittany B. Carson*, Jennifer M. Feenstra, Randall A. Dass, Petra Sekyrova, Ayuko Hoshino, Julian Petersen, Yuan Guo, Matthew M. Parks, Chad M. Kurylo, Jake E. Batchelder, Kristian Haller, Ayako Hashimoto, Helene Rundqivst, John S. Condeelis, C. David Allis, Denis Drygin, M. Angela Nieto, Michael Andäng, Piergiorgio Percipalle, Jonas Bergh, Igor Adameyko, Ann-Kristin Östlund Farrants, Johan Hartman, David Lyden, Kristian Pietras, Scott C. Blanchard, C. Theresa Vincent
Nature Communications, 2019, Vol.10, Issue 1, Article Number 2110

II. **Ribosome biogenesis during the epithelial-to-mesenchymal transition mediates a unique translation program**
Jake E. Batchelder, *Varsha Prakash*, Brittany Carson, Randall A. Dass, Matthew M. Parks, Chad M. Kurylo, Jennifer M. Feenstra, Johan Hartman, Jonas Bergh, C. Theresa Vincent and Scott C. Blanchard
Manuscript

III. **Wnt5a Signals through DVL1 to Repress Ribosomal DNA Transcription by RNA Polymerase I**
Randall A. Dass, Aishe A. Sarshad, Brittany B. Carson, Jennifer M. Feenstra, Amanpreet Kaur, Ales Obrdlik, Matthew M. Parks, *Varsha Prakash*, Damon K. Love, Kristian Pietras, Rosa Serra, Scott C. Blanchard, Piergiorgio Percipalle, Anthony M. C. Brown, C. Theresa Vincent
PLOS Genetics, 2016, Vol.12, Issue 8, Article Number e1006217

*Equal contribution

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LIST OF ABBREVIATIONS

4E-BP	Eukaryotic translation initiation factor 4E- binding protein
A site	Aminoacyl site
ADAM	A-disintegrin and metalloprotease
AKT	Protein kinase B
ALK	Activin receptor like kinase
AMH	Anti-müllerian hormone
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
BOP1	Block of proliferation 1
BRCA	Breast cancer gene
CaMK	Calmodulin-dependent protein kinases
CK1	Casein kinase 1
CPBF	Core promoter binding factor
DAG	Diacylglycerol
DCIS	Ductal carcinoma in situ
DENR	Density regulated re-initiation and release factor
DKC	Dyskerin
DVL	Dishevelled
ECM	Extracellular matrix
EdU	5-Ethynyl-2-deoxyuridine
eEF2	Eukaryotic elongation factor
EGF	Epidermal growth factor
EIBF	Enhancer I binding factor
eIF	Eukaryotic initiation factor
EMT	Epithelial-to-mesenchymal transition
ERα	Estrogen receptor alpha
eRF	Eukaryotic translation termination factor

ERK	Extracellular receptor kinase
EU	5-Ethynyl uridine
FBL	Fibrillarin
FGF	Fibroblast growth factor
FSP1	Fibroblast-specific protein 1
FUrd	5-Fluorouridine
FZD	Frizzled
GDF	Growth differentiation factor
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptors
GSK3β	Glycogen synthase kinase beta
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HERP	HES-related repressor protein
HES1	Hairy and enhancer of split 1
HIF-1α	Hypoxia-inducible factor 1 alpha
HnRNP	Heterogenous ribonucleoprotein
IGFR	Insulin growth factor receptor
ILC	Invasive lobular carcinoma
IP_3	Inositol triphosphate 3
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
JNK	Jun N-terminal kinase
LAP	Latency associated protein
LCIS	Lobular carcinoma in situ
LLC	Large latent complex
LOH	Loss of heterozygosity
LRP5/6	Lipoprotein receptor related protein 5/6
LTBP	Latent TGFβ binding protein
MAD1	MAX dimerization protein 1

MAML1	Mastermindlike 1
MAPKAP1	Mitogen-activated protein kinase associated protein 1
MAX	MYC associated factor X
MCT-1	Multiple copies in T-cell lymphoma 1
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MNK1	Mitogen-activated protein kinase interacting protein kinase 1
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NICD	Notch intracellular domain
NCL	Nucleolin
NFKβ	Nuclear factor kappa B
NOP56	Nuclear protein 56
NOR	Nucleolar organizer region
NPM	Nucleophoshmin
NST	No special type
P site	Peptidyl site
PCP	Planar cell polarity
PDK1	Phosphatidylinositol-dependent kinase
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
Pol I	RNA polymerase I
PR	Progesterone receptor
PtdIns	Phosphatidylinositol
PyMT	Polyoma middle T antigen
RACK1	Receptor for activated C kinase 1
RB	Retinoblastoma protein
rDNA	Ribosomal deoxyribonucleic acid

RPL	Ribosomal protein large subunit
RPS14	Ribosomal protein S14
rRNA	Ribosomal ribonucleic acid
Ser	Serine
SL1	Selective factor 1
SP-1	Specificity protein 1
TAF	TBP-associated factors
TBP	TATA-binding protein
TF-IIIB	Transcription factor IIIB
TGFβ	Transforming growth factor beta
THBS1	Thrombospondin 1
Thr	Threonine
TIF1A	Translation initiation factor 1A
TIP5	TTF-1-interacting protein 5
TNBC	Triple negative breast cancer
tRNA	Transfer ribonucleic acid
TSC2	Tubersclerosis complex 2
UBF	Upstream binding factor
UCE	Upstream control element
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 BREAST CANCER

Globally, cancer is the second leading cause of death with about 9.6 million deaths reported in 2018[1]. Tumorigenesis occurs when normal cells acquire the ability to proliferate without any restriction in growth control, in addition to acquiring abnormal morphology and functional properties that can initiate a tumor[2,3]. The hallmarks of cancer were initially described by Hannan and Weinberg, and further revised a decade later[2,4]. Apart from having endless proliferative capacity, tumor cells also find ways to evade immune responses, induce tumor inflammation, promote angiogenesis and acquire resistance to cell death programs[2,4]. Furthermore, cancer cells also reprogram cellular metabolism, escape growth suppression, and activate cellular programs that drive invasion and metastasis along with genome instability[2,4]. A benign tumor does not have invasive abilities whereas a malignant tumor can invade and spread to distinct organs in the body[2,5]. The three most common cancers worldwide are lung, breast and colorectal cancer and ranks within top five in terms of mortality[1]. While all three cancers make up for about one third cancer incidences globally, lung and breast cancer account for the majority of new cancer diagnoses[1].

Breast cancer is the most commonly diagnosed cancer in women and is the primary cause of mortality in women, followed by lung cancer[1]. In Sweden, breast cancer is the second most common cancer, with about 8017 new diagnoses reported in 2018[6]. The documented discovery of breast cancer dates back to about 3500 years ago where it was reported by Egyptians as 'tumors or ulcers of the breast'[7]. Normal breasts are composed of fat, connective tissue and glandular tissue called lobes[7]. A tumor that develops from the duct or lobular tissues of the breast is referred to as breast cancer[7]. These tumors contain highly proliferative cells due to genetic mutations, aberrant cell signaling, loss of tumor suppressors or over-activity of oncogenes[8].

1.1.1 Classification of breast cancer

Breast cancer is classified based on histopathological type, molecular status, stage, and grade[9]. The classification of breast cancer based on its histopathological and molecular status are the estrogen receptor alpha (ERα), the progesterone receptor (PR), the epidermal growth factor receptor (EGFR) HER2/neu, the tumor suppressor p53, the BRCA gene (BReast CAncer gene), loss of heterozygosity (LOH) and contributes to the main molecular markers used in the diagnosis of breast cancer [10]. The ERα and the PR status of a breast tumor will determine the type of primary line of therapy implemented in the clinic. ERα expression in breast tumors is

noted in about 70% of all breast tumors and denote the capacity of the tumors to respond to endocrine therapy[11,12]. PR is transcriptionally activated by ERα and is therefore correlated to the expression of ERα making it responsive to endocrine therapy[13]. HER2/neu is a growth promoting protein expressed in about 15% of the breast tumors[14,15]. The expression of HER2 was initially considered to be a poor prognostic marker[16,17]. However, development of targeted therapy against HER2 led to the evolvement of HER2 as a prognostic marker[16,17]. Breast tumors lacking ERα, PR and HER2 expression are identified as triple negative breast cancer (TNBC) and are known for their aggressiveness and tumor relapse due to lack of therapy[18]. Ki67 is another critical marker used in the diagnosis of breast cancer[19,20]. This protein is associated with proliferating cells and utilized to assess the proliferation rate of tumor cells. Breast tumors with low expression of Ki67 correlates with a better probability of survival in patients[19,20].

The Nottingham histological grading system or the Elston Ellis grade system is used to classify breast tumors based on their morphological and cytological validation[21]. In this system, breast cancers are classified as grades I to III based on the aggressiveness of the tumor[21]. Mitotic count, nuclear polymorphism and tubule formation are the main factors assessed by the pathologist[21]. Grade I breast cancers are low-grade tumors that are non-invasive with slow growth rates and are well differentiated[22]. Grade II tumors are of moderate aggressiveness with abnormally looking/dedifferentiated cells with slightly higher proliferation rate[22]. Grade III are high grade tumors with poor differentiation and high proliferation rates with chances of metastasis[23].

Breast cancer can also be classified based on the TNM (Tumor, Node, Metastasis) system, depending on 1) primary tumor characteristics 2) if the cells have spread to lymph nodes and 3) if metastases are found[24]. This gives rise to a five staged scale which reflects the severity of the disease, starting from stage 0 which is the non-invasive ductal carcinoma *in situ* and proceeds through stages I to IV denoting invasive breast cancer[24]. Stage 0 is when the breast cancer is non-invasive[24]. Stage I with sub-stages A and B, is when the tumor is less than 2 centimeters and has not spread beyond the breast itself even though it is invasive or if the tumor cells are present in the nearby lymph nodes with an absence of a primary tumor in the breast[24]. Stage II tumors (with sub-stages A and B) are invasive and have spread to the nearby and auxiliary lymph nodes with tumor size ranging from less than 2 centimeters to 5 centimetres[24]. Stage IV tumors are those that have metastasized to distant organs[24,25].

1.1.2 Non-invasive and invasive breast cancer

The majority of breast cancer arises from the epithelial lining of the duct[26] whereas tumors arising from the lobes constitute about 10-15%[9,26]. If the tumor cells are still confined in the ducts or lobules, the breast cancer is classified as non-invasive and benign[9,27]. The most common non-invasive breast tumor is ductal carcinoma in situ (DCIS), which is a premalignant lesion that can be further classified into low, intermediate and high grade based on their nuclear polymorphism and cellular attributes[28]. The lobular carcinoma *in situ* (LCIS) is the second most common type of non-invasive breast tumor and is often considered as a precursor of invasive carcinoma progression[29] (**Figure 1**).

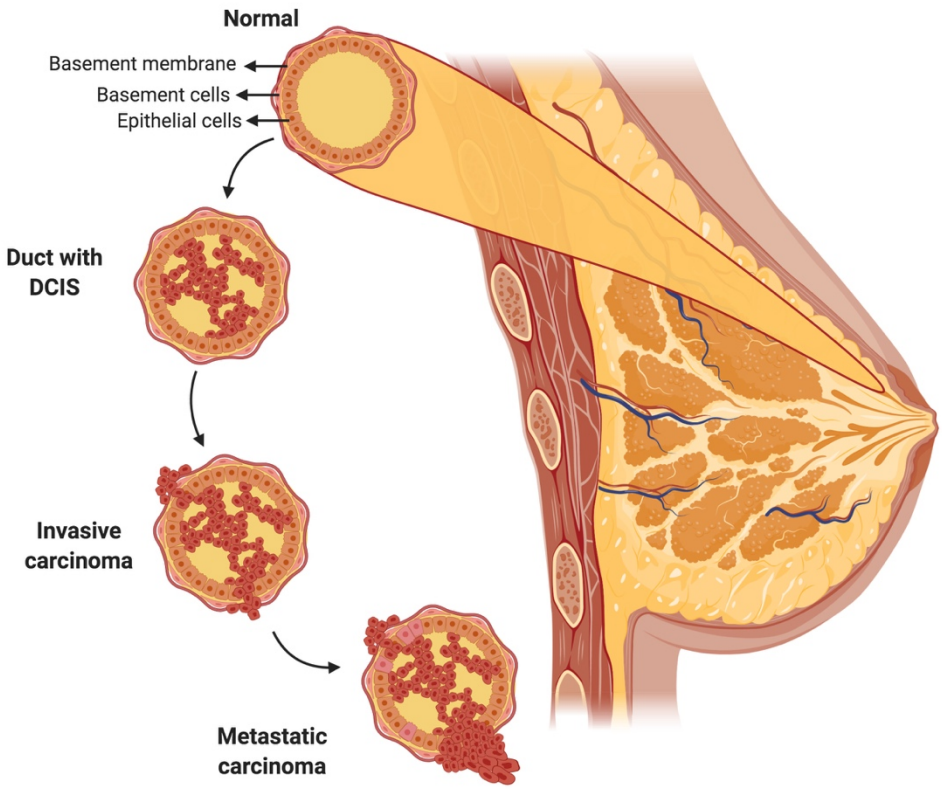

Figure 1. Schematic demonstrating sub-breast tissue origins of breast cancer and progression towards aggressiveness

Invasive malignant breast tumors have acquired the capacity to invade surrounding tissues and form secondary tumors, i.e., metastases in distant organs[27]. Invasive carcinoma of no special type (NST) is the most frequent type of invasive breast cancer and 80% of invasive carcinomas belong to this classification (this type was previously known as ductal carcinoma not otherwise specified)[27]. The second most common invasive breast cancer is invasive lobular carcinoma (ILC) and constitutes about 10% of the all invasive breast cancers[27].

As a breast tumor is relatively easy to surgically remove from the primary location, the invasion and metastasis formed by malignant tumor cells are the cause of over 90% of all cancer-related deaths [30,31]. Around 15% of patients with breast cancer develop metastases to distant sites in less than 3 years from diagnosis, though development of metastasis after 10 years of diagnosis also occurs[32]. Invasive breast cancer cells can disseminate and spread to distant sites forming metastasis most commonly in the lung, bone and liver[30,31]. Metastasis formation is a multistep cascade where the cancer cells invade surrounding tissues and enter the lymphatic or blood stream after acquiring invasive and migratory abilities[33]. These cells then form a secondary tumor at the metastatic site after extravasating through the endothelium[33]. A critical mechanism by which breast cancer cells acquire invasive ability is through epithelial-to-mesenchymal Transition (EMT), a cellular program that enables cells to leave the primary tissue[34].

1.2 EPITHELIAL–MESENCHYMAL TRANSITION

Epithelial-Mesenchymal Transition (EMT) is a physiological process known for its indispensable role in implantation, embryogenesis and organ development[35–37]. EMT is the process in which epithelial cells undergo biochemical and physical changes to acquire a mesenchymal phenotype[38]. Epithelial cells are non-mobile and adhere to one another and to the basal membrane[38]. However, mesenchymal cells have the capacity to migrate and invade due to the loss of cell adhesion, remodeling of the cytoskeleton and the expression of matrix metalloproteinases (MMP), which are capable of degrading the extracellular matrix[38]. In embryogenesis during gastrulation, EMT drives the separation of mesendoderm into mesoderm and endoderm in the primitive streak of the epiblast layer[35–37]. Mesoderm formed in between the epiblast and hypoblast layers then gives rise to the primary mesenchyme[35–37]. Moreover, EMT plays an important role in various pathological processes such as organ fibrosis and tumor invasiveness[38]. The role of EMT in organ fibrosis, e.g. kidney, is fairly well understood. Expression of fibroblast specific protein (FSP1) in the tubular epithelial cells correlates with EMT in kidney fibrosis[34,39]. These FSP1+ epithelial cells that acquire partial mesenchymal signatures, migrate to the interstitium of the kidney through the damaged basement membrane where they accumulate to gain a fibroblast phenotype after losing their epithelial signature completely[34,39].

Another important pathological implication of EMT is its role in tumor metastasis[34,39]. Although cell proliferation is the main hallmark of epithelial cancers, EMT has been implicated in the mechanisms leading to formation of secondary tumors at distant sites[39]. By enabling cell migration, EMT has been suggested to be the first step of the metastatic cascade[40].

A main characteristic of cancer cells undergoing EMT is that they lose the expression of the adhesion protein, E-cadherin. Regulation of the E-box elements in the E-cadherin promoter by the EMT associated transcription factors like Snail1, Snail2 and Twist leads to its transcriptional repression. Zeb1, 2 and basic helix-loop-helix (bHLH) factors also bind the E-cadherin promoter during EMT and represses its expression. Gain of mesenchymal proteins like Vimentin and N-cadherin are also observed during EMT. Induction of EMT requires coordination of many signaling pathways, which results in elevated expression of main EMT transcriptional regulators, Snail1 and Twist[41].

The Transforming growth factor beta (TGFβ) signaling pathway is one of the main inducers of EMT, which acts through subcellular signaling proteins known as Smads, which transduce signals from the cell surface to the cell nuclei[41]. In co-operation with TGFβ, the RAS pathway has also been shown to drive EMT, by reducing cell proliferation through Smad3 activation[42]. Additionally, the Wnt signaling pathway can induce EMT through its canonical signaling cascade wherein β-catenin is stabilized by glycogen synthase kinase-3 beta (GSK3β) inhibition[42]. β-catenin then translocate to the nucleus and regulates various transcription factors including expression of Snail1[42]. Hypoxia can trigger EMT through stabilization of HIF-1α, which in turn leads to the activation of Twist and Snail1 at the onset of EMT[42]. Transcriptional regulators of EMT work by both induction and repression[43]. Snail family primarily acts as repressors however recent literature also indicates that Snail1 can act as an activator[43]. Snail family consists of zinc-finger transcription factors which utilizes zinc to bind to DNA and regulates EMT both during development and tumor progression and metastasis[45]. Snail1 is shown to associate with Smad3 and Smad4 to regulate TGFβ induced EMT[44]. Tight junctional proteins like Claudin and Occludin are also repressed by Snail1 during EMT[46]. Snail1 also plays a role in the degradation of extra cellular matrix (ECM) which is mediated by matrix metallo-proteinases (MMPs)[45,47]. Snail1 contributes to the acquisition of invasive ability during EMT by contributing to cytoskeletal changes by upregulating RhoB and thereby modulating cell shape[45,47]. Snail1 is also involved in the regulation of cell cycle progression by halting cells at the G1/S transition as well as conferring resistance to cell death[48]. This is mediated by downregulating Cyclin D2 and inducing p21/Cip1[48]. Snail1 is activated and regulated by a number of signal transduction pathways including TGFβ, VEGF, EGF, FGF, HIF, WNT and NOTCH[47,49]. Transcriptional regulators of EMT with activating roles are AP-1, SP-1, NFKβ factors and β-catenin, that induce the expression of mesenchymal genes like Vimentin[50,51](**Figure 2**).

Importantly, EMT is also thought to promote therapeutic resistance, which represents a major challenge for effective treatment of metastatic breast cancer[52,53]. Hence, further understanding and characterization of the EMT is essential to be able to design novel cancer treatments aiming to inhibit metastasis[52,53]. One of the main strategies to target EMT has been to interfere with the signaling cascade responsible for the initiation of EMT[54,55]. This, however, might not be a useful intervention in the case when the metastatic process has already been initiated[54,55]. Therefore, targeting the mesenchymal phenotype and prevention of mesenchymal-to-epithelial transition (MET), a process where the disseminated cells acquire 'epithelial-like' phenotype required to form secondary tumors, seems equally important and should be considered as an effective alternative strategy.

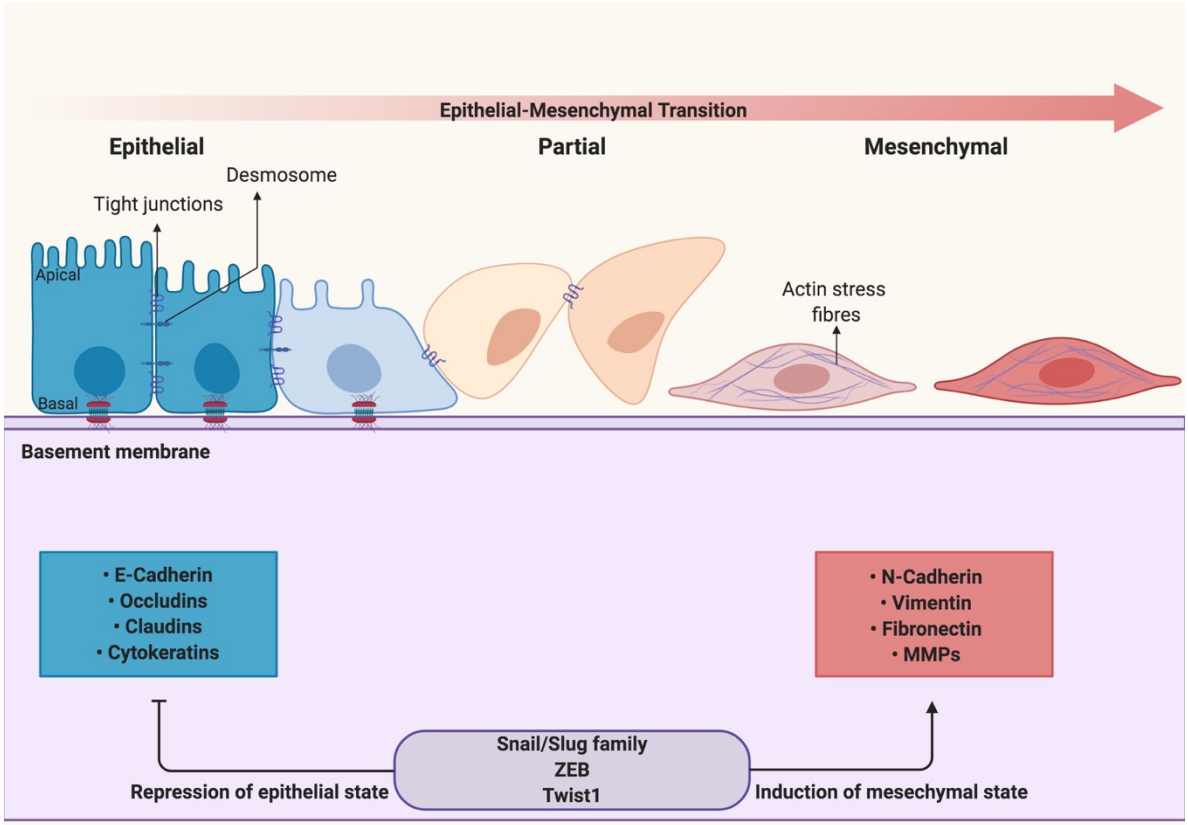

Figure 2. Schematic showing the process of epithelial-mesenchymal transition

1.2.1 The TGFβ signaling pathway

A principal regulator of EMT is TGFβ, which is a multifunctional cytokine found in all multicellular organisms and is conserved throughout evolution[56]. The TGFβ superfamily is comprised of TGFβs, activins, inhibins, bone morphogenetic proteins (BMPs), anti-müllerian hormone (AMH) as well as growth and differentiation factors (GDFs)[56]. The TGFβ family members are important regulators of several critical cellular processes like cell proliferation, differentiation, migration, invasion, and survival[56,57]. They are also involved in the regulation

of physiological processes, including embryonic development, EMT, extra-cellular matrix remodeling, angiogenesis, wound healing and immunosuppression[56,57]. Members of this superfamily are overexpressed in cancer, fibrosis and inflammation, where they affect cell growth, shape and migration thereby driving the disease progression[58].

The vertebrate genome encodes 30 ligands that belong to the TGFβ superfamily[59]. Abnormal expression of these ligands leads to developmental defects and progression of human diseases[60]. TGFβ1, TGFβ2 and TGFβ3 are the homologous isoforms of TGFβ in humans. These isoforms all signal in a similar manner and share the same receptor complex but differ in expression levels depending on the tissue in which they are expressed[61]. TGFβ ligands are initially produced as an inactive precursor and contained in a complex called the large latent complex (LLC). The LLC interacts with the latency-associated peptide (LAP) and a latent TGFβ-binding protein (LTBP) as a homo dimer[62,63]. In order for the TGFβ precursor to become activated, the LLC must be released from the extracellular matrix (ECM) leading to the subsequent release of TGFβ, making it available for binding to its receptors and for signaling. This release is mediated by the proteolysis of LAP[62,63] which in turn is facilitated through a number of different TGFβ activators, including MMP2 and MMP9 and thrombospondin 1 (THBS1)[62,63]. Proteolytic cleavage, increased interaction with integrins and pH changes can also activate latent TGFβ[62,63].

Once released from its inactive precursor state, TGFβ ligands are able to bind to the TGFβ receptor and initiate signaling[64]. Two main types of TGFβ receptors exists in humans: type I and type II receptors. Both of these receptors are serine/threonine kinase receptors that share related transmembrane regions and form a heterodimeric complex to enable TGFβ signaling[64]. The TGFβ family consists of seven type I receptors also called the activin receptor-like kinases (ALKs) and five type II receptors[62,64]. These receptors have different affinities for different ligands of the TGFβ superfamily[64]. For example, BMPs bind strongly to type I receptors and only weakly to type II receptors. Interestingly, the highest affinity of BMPs is seen in the combination of both receptors together[64].

After the ligand has bound the TGFβ receptor, the activation of TGFβ signaling is achieved either by the canonical pathway through Smads or by non-canonical pathway that is independent of Smads. These pathways can occur simultaneously[64]. Common Smads (Co-Smad), receptor Smads (R-Smads) and inhibitory Smads (I-Smads) are the three classifications of Smads[65]. During Smad dependent signaling, the R-Smads (Smad1,2,3,5) associates with Co-Smad (Smad4) and is translocated into the nucleus to activate or repress gene transcription depending on its binding partner[65]. Negative regulation of the TGFβ pathway is mediated by

Smad6 and Smad7 (I-Smads)[65]. PI3K-AKT, p38, RAS/ERK and JNK are non-Smad dependent signaling pathways mediated by TGFβ receptors[66]. The TGFβ signal transduction pathway has a dual role in cancer, exerting both tumor-suppressor and oncogenic signals through different factors which depends on the cellular context[67]. For example, TGFβ signaling has been shown to have a tumor suppressor effect during initial stages of the tumorigenesis process, and oncogenic effects as the cancer progresses, like promoting EMT[67] (**Figure 3**).

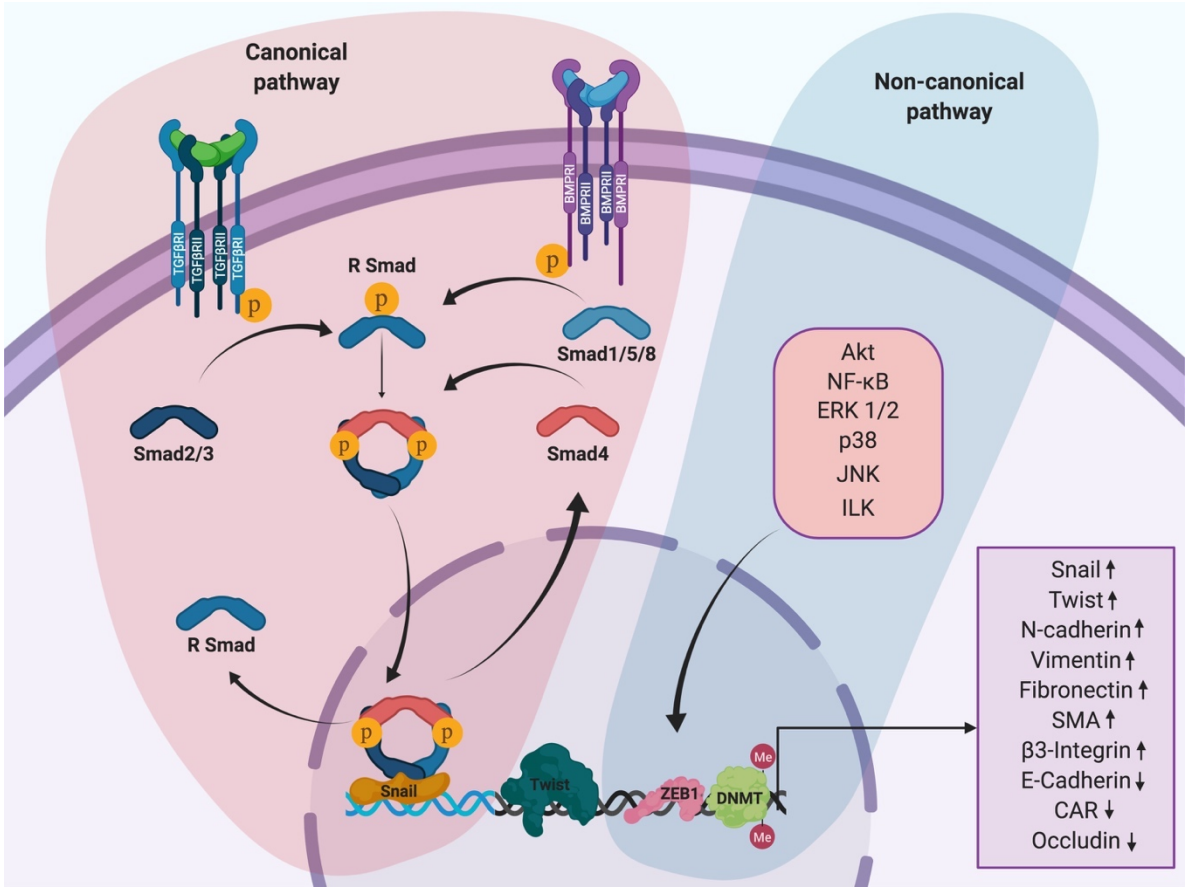

Figure 3. Diagrammatic illustration of TGF-β signal transduction pathway

1.2.2 The Wnt signaling pathway

The Wnt pathway is a highly evolutionary conserved signaling pathway that regulate critical cellular processes during development and tissue homeostasis, which frequently is dysregulated in human diseases. The Wnt proteins encoded by the Wnt genes (19 in mouse and human, 7 in *Drosophila* and 5 in *C. elegans*) act as ligands for the cell surface Frizzled receptors (Fzd), which initiates signaling that regulate a wide range of developmental functions including proliferation, migration, cell fate determination, cell polarity and stem cell maintenance [68]. The first Wnt gene was isolated in 1982 from mouse mammary tumor and was named int-1 (mouse mammary tumor integration site-1)[69]. It was subsequently discovered that the int-1 gene is

conserved across multiple species, with high similarity to the Drosophila wingless gene wg, which led to the name Wnt by combining wg and int[70].

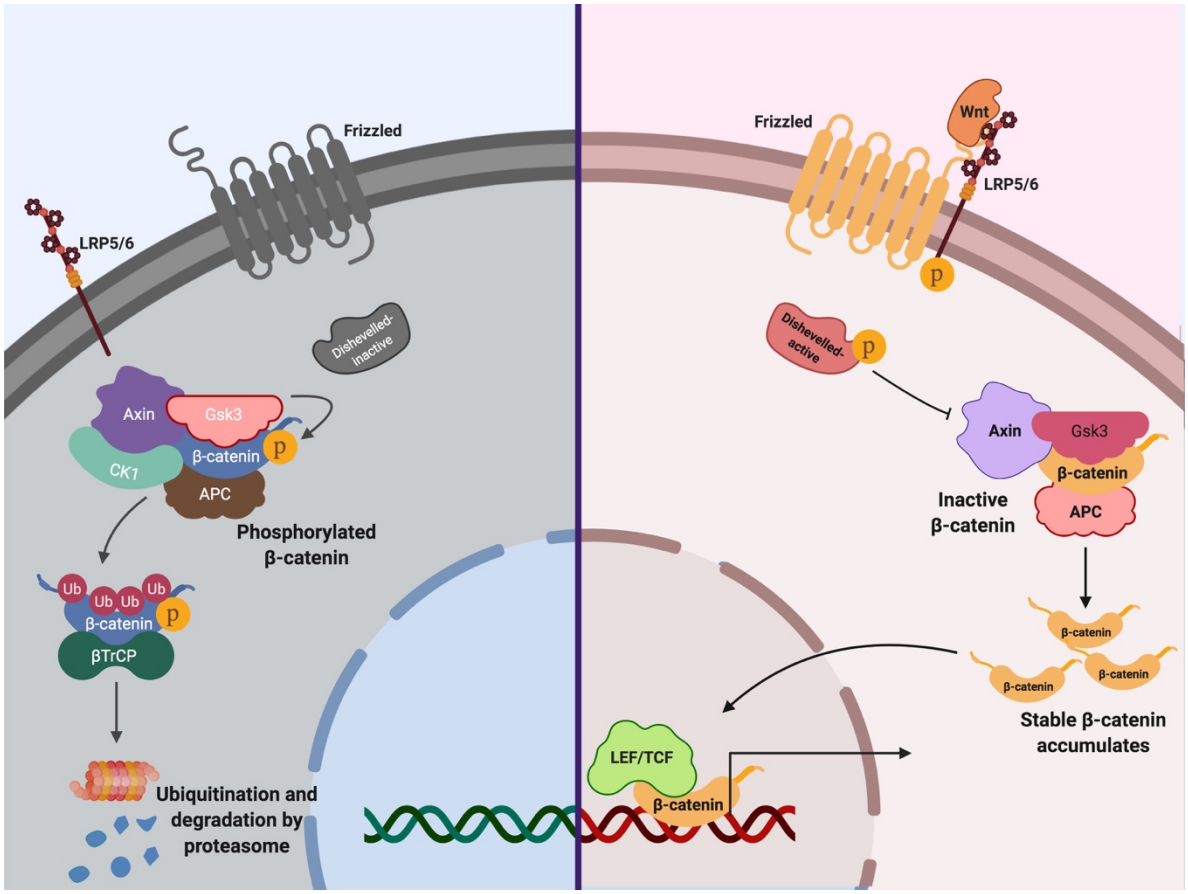

Figure 4. Diagrammatic illustration of canonical Wnt signaling pathway

Wnt signals through both β-catenin dependent (canonical) and β-catenin independent (non-canonical) pathways[70]. The canonical pathway, which mainly regulates cell proliferation, self-renewal, homeostasis and embryonic development, operates through β-catenin, which is present in both cytoplasm and adherent junctions of the cytoskeleton[70,71](**Figure 4**). When the Wnt pathway is inactive, cytoplasmic β-catenin is continuously phosphorylated and degraded by a destruction complex, composed of Axin, the tumor suppressor Adenomatous polyposis coli (APC), GSK-3β and Casein kinase 1 (CK1)[72]. When Wnt/β-catenin pathway is activated, Wnts bind to the Fzd receptors and low-density lipoprotein receptor related protein 5/6 (LRP5/6) resulting in the translocation of the scaffold protein Dishevelled (Dvl) from cytoplasm to plasma membrane to interact with Fzd[72]. The Axin/GSK-3β complex is then sequestered to plasma membrane and the GSK-3β and CK1 kinase phosphorylates LRP5/6 resulting in the inhibition of GSK-3β[73]. This in turn results in the accumulation of non-phosphorylated, stabilized β-catenin in the cytoplasm, which subsequently can translocate to

the nucleus and engage in transcriptional regulation of Wnt target genes[74,75]. Wnt target genes include cyclin D1[74,75].

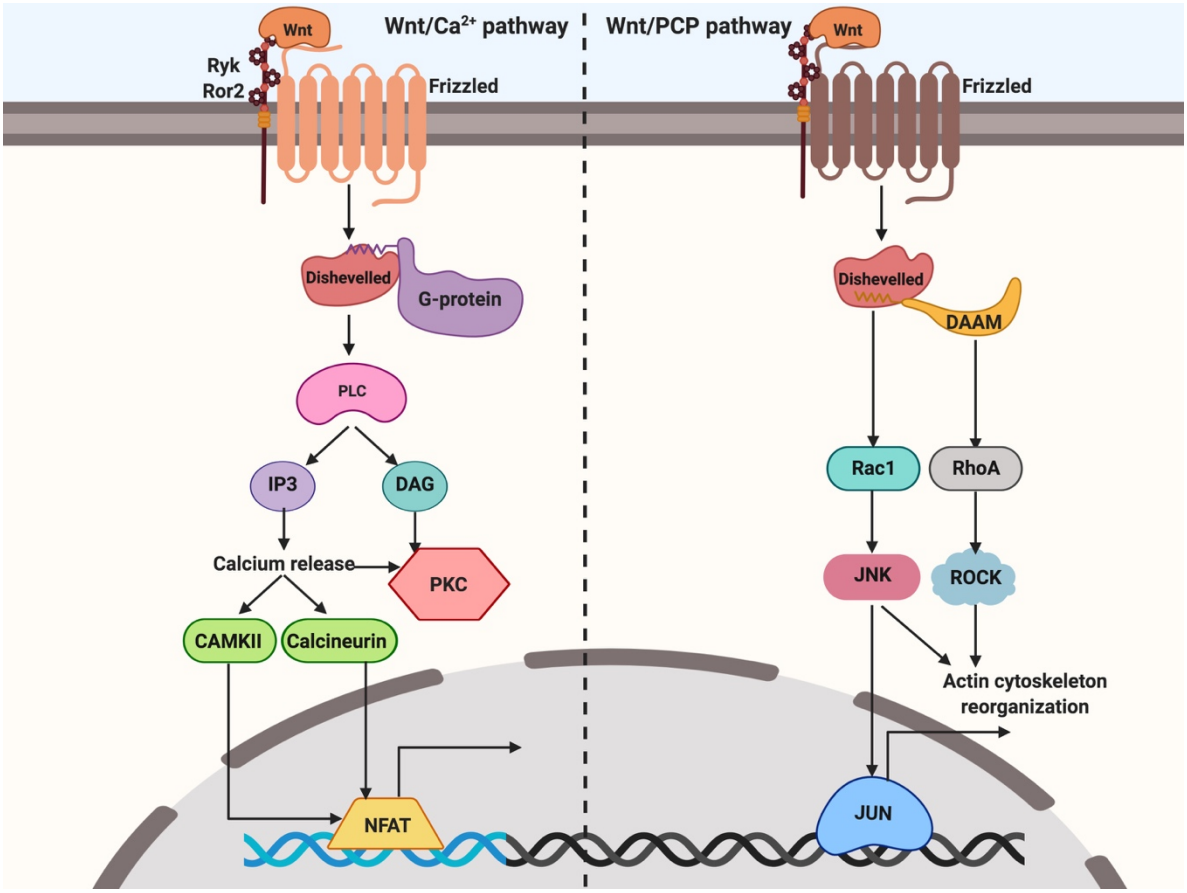

Figure 5. Diagrammatic illustration of non-canonical Wnt signaling pathway

The β-catenin-independent Wnt-signaling pathways (non-canonical) are less characterized than the canonical pathway and is initiated by Wnt11 and Wnt5a-binding to various receptors including ROR2[76](**Figure 5**). This pathway mainly regulates cell polarity, migration, motility and has been most studied in planar cell polarity (PCP) and intracellular calcium (Ca^{2+}) cascades[70]. In PCP, Wnt ligands bind to the Fzd independent of LRP and activates the PCP pathway, which is responsible for cell polarity and movement during neural crest migration in embryos[77]. In the Wnt-mediated Ca2+ cascades, heterotrimeric G-proteins are downstream of Wnt-Fzd signaling, leading to an increase in intracellular concentration of Ca^{2+}. This Wnt-mediated Ca2+ cascade also requires the activation of phospholipase C (PLC), which triggers diacylglycerol (DAG) and inositol triphosphate 3 (IP_3) synthesis, ultimately stimulating protein kinase C (PKC) and calmodulin-dependent protein kinases (CaMK)[78].

Mis-regulation of the canonical Wnt pathway has been widely associated with breast cancer development and activation of processes like cell proliferation, EMT and metastasis have been observed[68,76,79]. Particularly, in triple negative breast cancers, induction of canonical Wnt

signaling is observed, which correlates with a poor prognosis[80]. In contrast, the role of non-canonical Wnt signaling in cancer remains controversial as it has been suggested to exert both tumor suppressive and oncogenic effects. Non-canonical Wnts have been implicated to drive osteosarcoma[81] and epithelial-mesenchymal transition in melanomas by activating PKC[82]. However, downregulation of non-canonical Wnt5a signaling has also been demonstrated in a wide range of cancers like neuroblastoma, breast cancer, leukemia and colorectal cancer[83]. For example, Liu *et al* demonstrated that Wnt5a is frequently lost in hepatocellular carcinoma, suggesting a potential tumor suppressive role of this ligand[84]. Similarly, non-canonical Wnt signaling has been propsed to exert a tumor suppressive effect in breast cancer [85]. Thus, the role of Wnt signaling in development and disease is highly complex and varies depending on the cellular context[74] and needs further characterization.

1.2.3 The Notch signaling pathway

The Notch signaling pathway is an evolutionarily conserved cellular signaling pathway necessary for cell homeostasis through proliferation, apoptosis, cell fate specification, angiogenesis and homeostasis[86]. In vertebrates, the Notch signaling pathway encompasses four Notch receptors (Notch1, Notch2, Notch3, Notch4) and five ligands (Jagged1, Jagged2, Deltalike1, Deltalike3, Deltalike4). The Notch pathway is activated when a Notch ligand activates a receptor expressed on juxtaposed cells[87]. This activation is followed by a series of A Disintegrin and Metalloprotease (ADAM) and gamma-secretase dependent cleavages of the receptor, which results in the formation of an intracellular part of the Notch receptor, named the Notch intracellular domain (NICD)[88]. This intracellular fragment then translocates to the nucleus where it forms a complex with the transcription factor CSL (RBPJk), co-activators mastermindlike 1 (MAML1) and histone acetyl transferase (p300/CBP) and activates downstream target genes. These genes includes the Notch like hairy enhancer of split (Hes), Hes-related repressor protein (Herp), p21 and Myc[88].

Aberrant signaling of the Notch pathway has been identified in the pathology of several diseases, including cancer[89]. For example, dysregulated Notch signaling is highly implicated to drive certain hematopoietic cancers and solid tumors, including breast cancer[90–92]. Moreover, dysregulated Notch signaling have been found to drive EMT through hypoxia and estrogen signals from the microenvironment[93]. During hypoxia-induced EMT, NICD is stabilized by the hypoxia-induced transcription factor HIF-1α, resulting in increased Snail1 transcription[94]. In addition to this mechanism, HIF-1α can activate Snail1 and Snail2 by binding directly to the Hes1 and His2 promoters along with the Notch co-activator MAML1 during hypoxia[95].

1.3 BREAST CANCER TREATMENT STRATEGIES

Breast cancer is a heterogenous disease with multiple molecular subtypes. The ERα positive breast tumors makes up about 70% of all breast tumors[11]. Hence, endocrine therapy strategies targeting estrogen signaling has been critical in treating breast cancer for more than a century[96]. Competitive inhibitors of the ER binding sites have been in use since 1970s[96]. Downstream estrogen activity inhibitors like aromatase inhibitors includes exesmestane and anastrozole, prevents the catalyzation of androgen hormone into estrogens[97,98]. Fulvestrant, an ER antagonist which aids in degrading ER, has also been developed[99,100]. One of the primary lines of breast cancer therapy of ERα positive breast cancers is Tamoxifen, an estrogen receptor modulator (SERM) that has been shown to significantly increase the overall survival in patients[96]. The 'HER2 enriched' tumors that form about 20% of the breast tumors are treated with drugs like lapatinib, trastuzumab and pertuzumab that directly target the HER2 protein expression[101]. HER2 functions are mainly mediated through pathways like PI3K-AKT and RAS-MAPK, so targeting these pathways are potent therapeutic avenues[102,103]. PI3K mutations are found in over 20% of the breast cancer patients[104]. mTOR (mammalian target of rapamycin) is activated by phosphorylated AKT leading to cell proliferation and increased protein synthesis, making mTOR inhibition a therapeutic target[105]. Buparlisib is a PI3K-AKT inhibitor currently used in clinical trials. Everolimus, an mTOR inhibitor has already been approved for treatment of metastatic breast cancer in combination with exemestane, an endocrine therapy which have been demonstrated to result in an increase in the progression-free survival of the patients[106,107].

Chemotherapy is a treatment option when the endocrine targeting options have been exhausted or if immediate response is required[108]. Pre-operative chemotherapy has been shown to have equal outcome in regards to progression free survival and overall survival as compared to postoperative chemotherapy[108]. Anthracyclines are active chemotherapeutic agents that work by inducing apoptosis and inhibiting DNA and RNA synthesis whereas taxanes inhibit cell proliferation and function by stabilization of microtubules[109]. Anthracyclines and taxanes, in combination or in series are administered as current standard of care in early breast cancer[108]. Chemotherapy is considered preferable for TNBC and in HER2 positive patients following neoadjuvant therapy and surgical intervention[108]. The TNBC and HER2 positive subtypes show better response with patient outcome in relation to pathological response[108].

Ribosome biogenesis and dysregulation of its associated factors are frequently encountered in breast cancer[110]. Induction of ribosome biogenesis is observed in TNBC and inactivation of ribosomal protein RPL5-uL18 has been observed in 34% of breast cancers[110]. RPL5-uL18 has

a critical role in MDM2 inactivation together with 5S rRNA[110]. Highly proliferative, BRCA1/2 deficient tumors have been shown to be specifically sensitive to RNA Polymerase I (Pol I) inhibitors like CX-5461 and CX-3543 that inhibit the initiation of ribosome biogenesis by targeting the Pol I complex. The suggested mechanism for this is that these tumors cells have a decreased ability to repair DNA damage caused by blockage of replication forks by the ribosome biogenesis inhibitors[111]. This is in turn suggested to be due to G4 stabilization caused by the Pol I inhibitors[111]. CX-5461 is currently under advanced phase I clinical trials in Canada (NCT02719977)[111]. The trial includes breast cancer patients carrying the BRCA1/2 deficient tumors[111]. Taken together, targeting of ribosome biogenesis may therefore represent a novel potent therapeutic target which is distinct from ionizing radiation and conventional chemotherapy. There is therefore, a need for more thorough biological characterization of ribosome biogenesis in tumor progression to know how we can better target this complex and metabolic pathway.

1.4 RIBOSOME BIOGENESIS AND ITS REGULATION

Ribosomes are complex molecular machines that are responsible for protein translation[112]. They are essential for cellular growth and proliferation and the target of many regulatory signaling pathways[112]. Ribosomes play a central role during normal growth, development and defects in ribosome synthesis will cause deleterious effects on cell physiology[113]. The production of new ribosomes, known as ribosomal biogenesis, is a complex process involving transcription of ribosomal RNA (rRNA) genes by RNA Polymerases (Pol I and Pol III), cleavage of the pre-rRNA transcripts, followed by rRNA modifications (methylation and pseudouridylation[114]) and assembly with ribosomal proteins (rp) synthesized by RNA Polymerase II (Pol II), to form the mature ribosomes[115](**Figure 6**). The ribosome backbone consists of rRNA which is transcribed in the nucleolus, the most prominent structure in the nucleus[116]. The human genome is estimated to have on average 200-400 copies of rRNA genes while there are individuals that encompass more than 1000 copies that are transcribed with high efficiency to generate sufficient ribosomes to fulfil the demands of proliferating cell[115].

rRNA biogenesis starts with that ribosomal DNA (rDNA) is transcribed by Pol I as a large 47S rRNA transcript in humans, and 45S in mice[117]. This transcript is further cleaved into 18S, 5.8S and 28S transcripts, which form the rRNA backbone of the ribosomes along with the 5S transcript synthesized by Pol III[117]. rDNA transcription is initiated by the assembly of a multi-protein complex at the rDNA promoter along with various auxiliary proteins[117]. Specifically, the upstream Binding factor (UBF) and the Selectivity Factor 1 (SL1) complex are the main Pol I specific components that act synergistically to form the pre-initiation complex necessary

for initiation of rDNA transcription[117]. UBF is a DNA binding protein that recognizes both DNA Control elements, CORE and Upstream control element, UCE[117]. UBF is the first Pol I specific transcription factor identified and has repeated regions of 85 amino acids homologous to high mobility group proteins (HMG boxes). This protein bind promoters and facilitate bending in DNA to activate transcription[117]. In addition, these proteins have been suggested to activate chromatin throughout the rDNA gene cassette to facilitate transcription elongation, since UBF and SL1 are found not only at the promoter site but also along the transcribed region[117]. Another known important regulator of rDNA transcription is the SL1 complex, which exists as a TBP-TAF1 complex in cells[118]. There are three different TAFs (TBP-associated factor) named according to their respective molecular mass of 48 (TAFI48), 63 (TAFI63) and 110 kDa (TAFI110)[119,120]. Together, the SL1 complex and Pol I can initiate basal transcriptional activity of the rDNA promoter, but for full activation of rDNA transcription, UBF binding is also needed[121]. Accordingly, polymorphisms that destroy the association of UBF with either the rDNA promoter or SL1 deteriorate the assembly of the Pol I complex and transcription activity of Pol I[122–124]. Other examples of known regulators of rDNA transcription are enhancer I binding factor (EIBF), which interacts with the proximal promoter and the enhancer region[125,126], Core promoter-binding factor (CPBF), which binds to the promoter and increases transcription efficiency by interacting with EIBF[127,128], and Topoisomerase I, which facilitates rRNA synthesis through DNA unwinding[117].

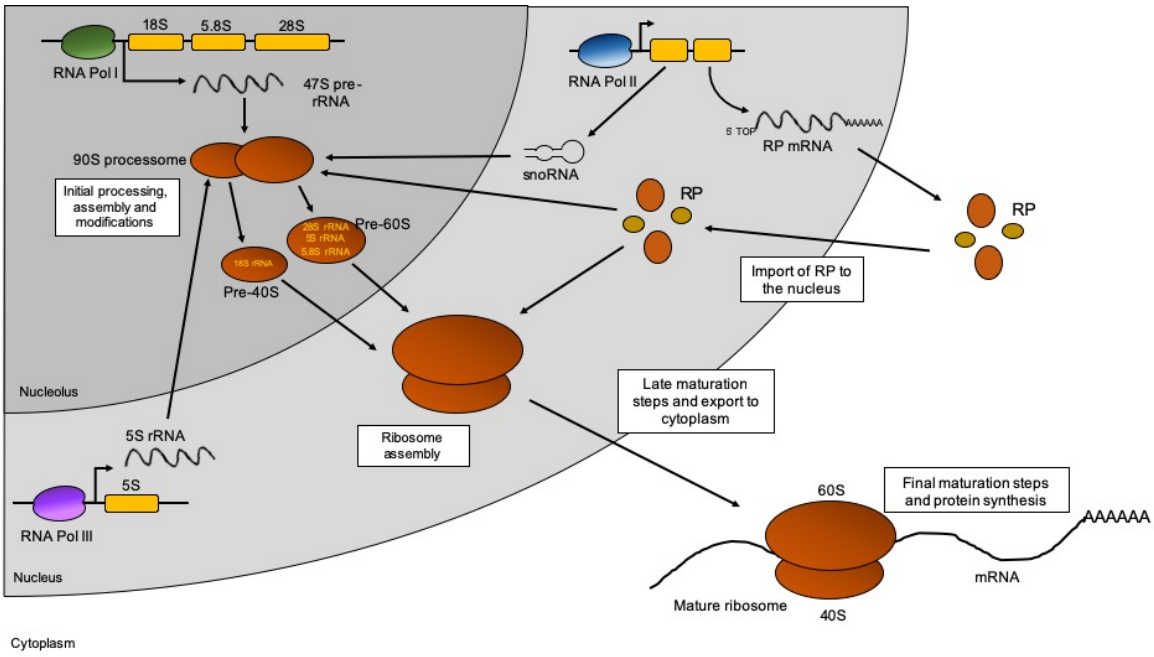

Figure 6. Illustration of the process of ribosome biogenesis

Post-translational modifications and in particular phosphorylation of the auxiliary proteins involved in ribosomal biogenesis play an important role in the regulation of rDNA transcription [129,130]. For example, the serine residues at the carboxy terminal activation sites of UBF have been found to be subjected to phosphorylation[129,130], which is essential for SL1 recruitment[131] and as such increases rDNA transcription activity. Therefore, increased UBF phosphorylation is observed when rDNA transcription is enhanced, for example, when quiescent cells are stimulated with serum[129,130], or during phytohemagglutinin induced lymphocyte stimulation[132]. SL1 is also known to be regulated by phosphorylation[133] and repression of rDNA transcription due to phosphorylation of SL1 is observed during mitosis[117]. In addition to phosphorylation, protein acetylation also positively regulates rDNA transcription[117], as Muth *et al* and Pelletier *et al* showed that UBF and SL1 subunits are acetylated *in vivo* during rDNA transcription[134].

Ribosome biogenesis is regulated by evolutionary conserved signaling pathways, including PI3-AKT, mTOR, MYC, and p53-Rb. Changes in nucleolar architecture and its association to cancer progression have been studied since the late 19th century[116]. Increased size of nucleolar organizing regions (NORs), which are visualized by silver stain, has been a classic feature of cancer cells and has been suggested as a potent prognostic factor[135]. The regulation of biogenesis by known oncogenes and tumor suppressors (mTOR, MYC, and p53-Rb) underlines the importance of future studies on the impact of this process on cancer initiation and progression[110].

1.4.1 The PI3K-AKT-mTOR signaling pathway

The PI3K-AKT-mTOR signaling pathway has been extensively studied due to its engagement in cellular processes like cell growth and proliferation which is ultimately linked to its role in regulating ribosome biogenesis[136,137]. The pathway consists of the three conserved kinases; mTOR, AKT (protein kinase B) and PI3K (phosphoinositide 3-kinase)[136,137]. AKT exerts its role in cell survival by phosphorylating a variety of substrates and is a downstream effector of the PI3K transduction cascade that modulates ribosome biogenesis. PI3K mediated activation of ribosome biogenesis is mediated by an induction of Pol I and Pol III mediated transcription[138]. Out of the many substrates of AKT that has been studied, the most important and well characterized substrate being linked to ribosome biogenesis is the mTORC1/2 complex[139,140]. mTOR is a kinase consisting of two complexes; the mTOR complex 1(mTORC1) or the mTOR complex 2 (mTORC2)[139]. The mTORC1 and mTORC2 complexes have common protein members; the mTOR, mLST8 or GβL (G protein β subunit like) and Deptor which associates with mTOR in the DEP domain[139]. The mTORC1 complex consists of Raptor whereas the mTORC2 complex consists of PRR5 (Proline rich protein 5), Sin1

(MAPKAP1) and Rictor [139]. mTORC2 is actively involved in cytoskeleton remodeling and in the regulation of ribosome biogenesis by the physical association of mTORC2 with the mature ribosome whereas mTORC1 is majorly involved in regulation of ribosome biogenesis and translation in proliferating cells[139,141](**Figure 7**).

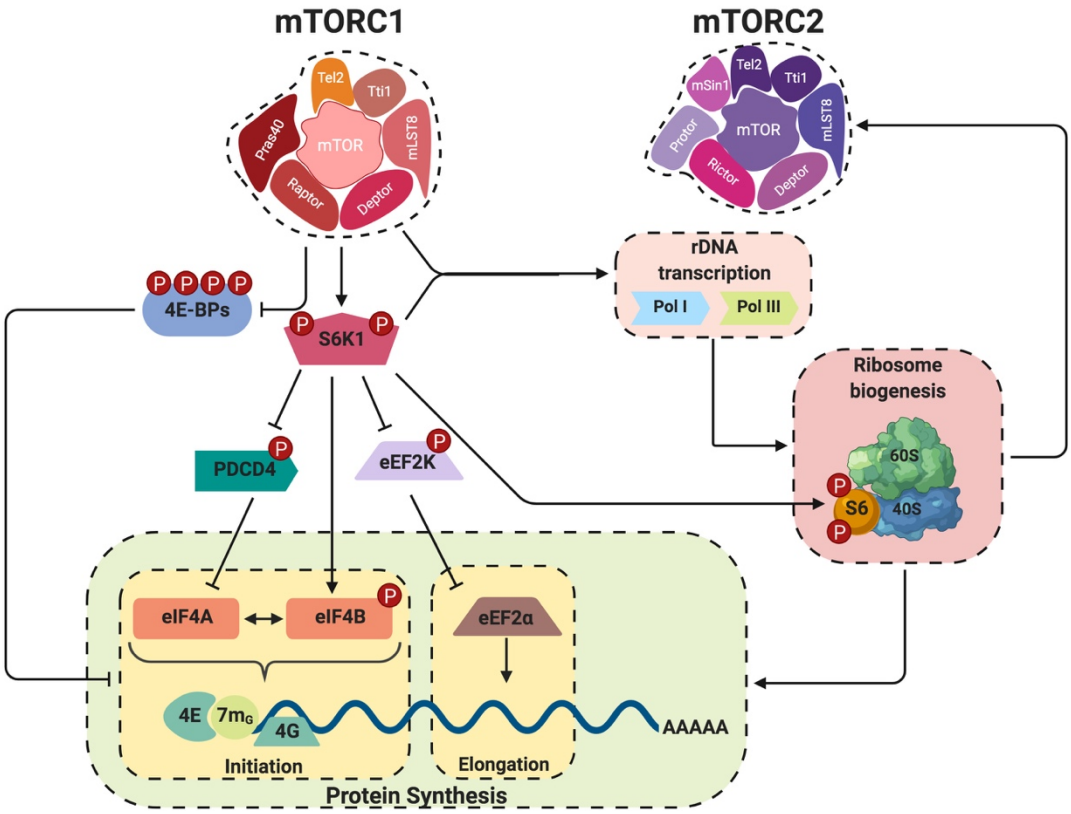

Figure 7. Schematic representation of mTORC regulation of ribosome biogenesis and translation

A role of mTOR in rDNA transcription has been documented where mTOR is involved in the activation of UBF and TIF-1A. mTOR regulates the phosphorylation of UBF at the C-terminus through ribosomal protein S6 kinase (S6K)[142]. Pol III dependent 5s rRNA transcription is also activated after recruitment of mTOR to its promoter resulting in the phosphorylation of MAF1 which inhibits TF-IIIB complex assembly and transcription initiation[143]. Further, mTORC1-AKT mediated phosphorylation of the transcriptional repressor MAD1 releases MAD1 from the MYC-MAX complex which can then engage in rDNA transcription[144].

1.4.2 Role of Protein kinase CK2 in ribosome biogenesis

Protein kinase CK2 that plays a critical role in tumor development, cell cycle progression, regulation of oncogenes and tumor suppressors[145]. It is a serine threonine kinase and regulates ribosome biogenesis by modulating the interaction of UBF, SL1/TIF1B, TIF-1A, NPM and

NCL with the Pol I complex through phosphorylation thereby affecting rDNA transcription[145,146]. CK2 mediated phosphorylation leads to the stabilization of UBF-SL1/TIF1B complex as well as its binding to rDNA[147]. In addition, CK2 also binds to the initiation inducing sub-component of Pol I, Pol Iβ[118].

1.4.3 The MAPK signaling pathway

Mitogen activated protein kinases (MAPK) signaling pathway are known to regulate ribosome biogenesis through the two critical factors involved in Pol I mediated rDNA transcription, UBF and TIF-1A[148,149]. The MAPK family member extracellular signal-regulated kinase (ERK), promotes UBF-DNA interaction by modulating its phosphorylation leading to increased Pol I engagement at the promoter[150]. In addition, ribosomal S6 kinase, another member of the MAPK family, is involved in the phosphorylation of TIF-1A contributing to regulation of ribosome biogenesis[148,150].

1.4.4 Role of MYC in ribosome biogenesis

MYC is a proto-oncogene that regulates all three RNA polymerases and is associated with regulation of at least 15% of the genome[151]. Three genes belong to the MYC family: MYC, MYCN and MYCL1, which all are transcription factors regulating the expression of genes involved in cell growth, metabolism, apoptosis, DNA repair, angiogenesis and protein synthesis[152]. MYC has been shown to repress and activate genes by chromatin remodeling. MYC's role in chromatin remodeling, transcriptional changes and ribosome biogenesis plays a significant role in cell physiology like proliferation, senescence and differentiation[152].

The role of MYC in regulation of ribosome biogenesis is well documented[151]. MYC, for example, plays a direct role in influencing rDNA transcription by modulating recruitment of Pol I complex components (UBF and SL1) to the rDNA promoter[151]. Moreover, UBF expression is controlled by MYC in Pol II dependent manner. Regulation by increased binding of Pol I complex is executed through MYC binding to TBP and TAFs of SL1[151]. Additionally, MYC enhances rDNA transcription by increasing the recruitment of co-factors and remodeling of the chromatin at rDNA loci[151].

The role of MYC in regulating the transcription of Pol III transcribed 5S rRNA is through TFIIIB and by expression of ribosomal proteins transcribed by Pol II further supports MYC's key regulatory role in ribosome biogenesis and as a pan RNA polymerase regulator[151,153].

Proteins involved in the processing of rRNA like fibrillarin (FBL), nucleolin (NCL), nucleophosmin (NPM), block of proliferation1 (BOP1), dyskerin (DKC), nuclear protein 56

(NOP56) are also regulated by MYC[154]. NPM1 is involved in direct regulation of ribosome biogenesis by direct association with MYC at target promoters[155]. NPM1 is a nuclear phosphoprotein which positively regulates ribosome biogenesis and contributes to protein synthesis[155]. It also regulates ribosomal protein nuclear import, ribosomal subunit export and assembly of ribosomes[156].

1.4.5 Regulation of ribosome biogenesis during cell cycle

Cell cycle regulators play a crucial role in the regulation of ribosome biogenesis since the balance between ribosome biogenesis and cell cycle progression is essential for normal cell survival and growth[144]. The retinoblastoma (Rb) tumor suppressor is the key G1 phase checkpoint regulator of cell cycle progression before entering into S phase[157]. When the cell is ready to divide, Rb gets phosphorylated, leading to its inactivation and hence allows cell cycle progression[157]. Rb in its unphosphorylated form regulates cell cycle progression by interacting with transcription factor E2F to activate downstream gene targets[157]. Rb is phosphorylated and inhibited in proliferating cells by cyclin-dependent kinases like CDK2, CDK4 and CDK6[158]. Hyper-phosphorylation of Rb leads to a loss in the activity of Rb by activation of these cyclin dependent kinases[158]. A direct link between ribosome biogenesis and cell cycle is that the hyper-phosphorylated, non-active form of Rb interacts with UBF which is a part of the Pol I complex to inhibit the synthesis of 47S transcript[110]. Similarly, synthesis of 5S rRNA is impaired by association of inactive Rb with TF-IIIB of the Pol III complex[110].

Two other important regulator of rRNA biogenesis and the cell cycle is the p14/p19^{ARF} (ARF), which is encoded by the CDKN2A gene and p53[159,160]. p53 is a major inducer of cycle arrest and/or apoptosis and is mutated in more than 50% of all human tumors, whereas ARF mutations are found in about 40% of the human tumors[156,161]. ARF represses ribosome biogenesis in a p53 independent manner by affecting rRNA processing by binding NPM1 and promoting degradation by ubiquitinylation[162]. NPM1 also associates with Mouse double minute 2 (MDM2) which is a E3 ubiquitin ligase responsible for the degradation of p53[163]. This association leads to the inhibition of MDM2 activity leading to the stabilization of p53 in the nucleus[163]. p53 regulates ribosomal biogenesis by binding to SL-1 and transcription factor IIIB (TFIIIB) interfering with the transcription of rRNAs, snoRNAs and tRNAs[120,153]. Moreover, the auto-regulation of p53, where it initiates the transcript synthesis upon MDM2 binding for degradation, is a checkpoint for ribosomal stress[164,165]. Like NPM1, nucleostemin (NS) also results in the stabilization of p53 by binding to MDM2[166]. Ribosomal proteins like RPL5, RPL11, RPL23, RPS3 and RPS7 bind directly to MDM2 thereby inhibiting p53[164,165,167]. Marechal et al co-immunoprecipitated RPL5 and 5S rRNA along with the p53-MDM2

complex, suggesting its role in the regulation of ribosome biogenesis or translation of cell cycle specific mRNAs[168]. It has been shown that ribosomal proteins not assembled into ribosomes activates p53 by binding to MDM2 and inhibiting it. RPS14 is found to inhibit CDK4/Cyclin D1 complex and activate Rb by inhibiting its phosphorylation leading to senescence and cell cycle arrest[158]. Pestov et al suggests that the cell cycle regulatory pathway might send cues that regulate ribosome biogenesis[169]. Collectively, these data show an intimate link between ribosome biogenesis and cell cycle regulation.

1.5 TRANSLATIONAL MACHINERY AND ITS DYSREGULATION IN CANCER

1.5.1 Translation Initiation

The process of translation takes place through the three main stages of initiation, elongation, and termination[170]. The main factors involved in initiation are eukaryotic initiation factors (eIF) 1,2,3,4,5 along with the ternary complex made of eIF2, GTP and Met-tRNAi[171,172]. These initiation factors aid in the formation of the 43S pre-initiation complex together with 40S ribosomal subunit, which then interacts with m^7Gppp capped 5′ end of mRNA during initiation[172]. This loading of the 43S pre-initiation complex to the 5' untranslated region (UTR) requires co-operation of co-factors like eIF4F, eIF4B and eIF4H. eIF4F consists of the DEAD-box RNA helicase eIF4A and eIF4G. The latter binds the eIF4E, eIF4A, poly(A)-binding protein and eIF3 in addition to the cap binding protein eIF4E[172,173]. eIF4B and eIF4H plays a role in inducing the helicase activity of eIF4A[174]. eIF4E bound to eIF4F independent of its 5' cap binding activity, also enhances the helicase activity of eIF4A[175]. The pre-initiation complex runs through the triplet codons entering the ribosome at P(peptidyl) decoding site complementary to the anticodon Met-$tRNA_i$, checking its integrity downstream of 5'UTR[172]. eIF1 and eIF1A recognizes the start site (AUG) of the mRNA by modifying the mRNA-binding cleft structure[172]. The translation process is initiated when the 80S ribosomal unit, formed by the 60S ribosomal subunit and start codon complex is assembled on the mRNA after release of the initiation factors[172]. The A-site (aminoacyl) is then prepared to be filled with the complementary tRNA (aminoacyl) received by the 80S complex which leads to the first peptide bond synthesis[172,176]. 5′ 7-methylguanosine (m^7G) cap independent translation of mRNAs are seen in some eukaryotes where the 40S subunit is recruited to the internal ribosome entry site (IRES), which is a cis-acting element situated at the 5' UTR[172]. IRES *trans*-acting factors (ITAFs) are involved in the regulation of IRES and their interaction with the 40S subunit directs the initiation codon to the ribosomal P-site. IRES mediated translation is upregulated under stress conditions when cap dependent translation is inhibited[177]. Modulation of IRES

mediated translation is also dependent on the ribosome composition changes in addition to ITAF activity[177].

1.5.2 Translation elongation

The two main factors involved in the execution of the translation elongation process are eEF1 and eEF2, which are responsible for the delivery of aminoacyl tRNA to the A site of the ribosomes and for aiding ribosome translocation[178]. The GTP from the eEF1A- GTP complex is hydrolyzed upon codon matching after the aminoacyl tRNA delivery to the A site resulting in the release of eEF1A-GDP complex, that is then recycled by eEF1B[179]. Unphosphorylated eEF2 is mainly involved in ribosome translocation where the peptide is transferred from the A site to the P site of the translating ribosome[179]. eEF2 Kinase (eEF2K) can phosphorylate eEF2 resulting in inhibition of its activity and potentially binding thereby perturbing the elongation process[179]. During elongation, the ribosomes move along the mRNA in a stop-and-go fashion and is not consistent, with various cis and trans factors influencing the ribosome speed along the mRNA[173].

1.5.3 Translation termination

When the ribosomal A site encounters a stop codon on the RNA, translation termination occurs via the release of eRF1 and eRF3[180,181]. eRF1 is responsible for the recognition of the stop codon and the release of tRNA while eRF1 stimulates the release of peptide from the complex[182]. The 40S subunit is, in some cases, found to be associated with mRNA after a mature polypeptide has been released, which enables re-initiation of translation from a new start codon downstream of the previous stop codon[180]. Re-initiation during translation is observed in 3'UTRs which are upregulated during conditions of stress arising from nutrient starvation. A non-canonical initiation factor complex MCT-1/DENR is also implicated in the re-initiation process in addition to the standard factors[183].

1.5.4 Dysregulation of factors involved in translation in cancer

Regulation of mRNA transcription has been widely studied in cancer. During recent years studies have revealed that mRNA transcript levels and proteins levels are not always mirroring each other and therefore highlights the importance of gene regulation at a translational level[184]. Importantly, the translation machinery components have also been found to be highly dysregulated in cancer[185]. The role of initiation factor eIF4E in tumorigenesis is well studied[185]. One study has demonstrated that increased expression of eIF4E results in increased translation of genes that induce tumorigenesis including the ones responsible for ribosome biogenesis, apoptosis, cell signaling, oxidative phosphorylation and nucleotide biosynthesis[186].

Phosphorylation of eIF4E, which is carried out by the MNK1/2 serine/threonine kinases, is also associated with enhanced translation of tumor inducing mRNAs and is also critical for EMT[187–189]. Upregulation of HuR (ELAV-like RNA-binding protein 1), one of the components required for eIF4E mRNA stability regulation in cancer, leads to induced eIF4E protein levels and is implicated in gene dysregulation at transcriptional and translational level by translational upregulation of specific tumorigenic mRNAs, HuR and eIF4E[190]. Another example is the modulation of translation process by the mTORC1 complex through regulation of eIF4F assembly[191,192]. Specific mRNA translation and global protein synthesis upregulation by mTORC1 is mediated downstream through activation of 4E-BPs and ribosomal protein S6 kinases (S6Ks) [191–193]. The phosphorylation of 4E-BP by mTORC1 plays a role in its binding competence with eIF4G for the eIF4E binding site, thereby, regulating the eIF4F complex formation[191–193]. The eIF4F complex is essential to recruit 40S ribosomal subunit required for CAP-dependent translation[194]. This recruitment is made to the mRNA's 5′ N^7-methyl guanosine CAP [m^7G(5')ppp(5')N] in order to carry out CAP-dependent translation[194]. CAP-dependent translation starts off by the association of eIF4E to a modified guanosine called CAP at 5' end of the mRNA and is facilitated by the phosphorylation of 4E-BP by mTORC1 which leads to the release of eIF4E to form a complex eIF4F along with eIF4G[192,195]. In the absence of phosphorylation by mTORC1, 4E-BP remains bound to eIF4E thereby dissociating it from eIF4G[193]. The activation of p70S6K through phosphorylation by mTORC1 also enhances CAP-dependent translation by phosphorylating PDCD4 and eIF4B[193,196]. The activity levels of the mTORC1 and mTORC2 complexes are regulated by mTOR through phosphorylation of its components. Another example is that cancer cells express higher levels of phosphorylated eIF2α, which induces the translation of mRNA genes specific for stress adaptation, resulting in increased survival of tumor cells[197].

Furthermore, structural features of mRNA like the 5'UTR and 3'UTR lengths and its stability contributes to its translational regulation and its association with miRNAs, initiation factors and RNA binding proteins[198]. These features are more commonly found in mRNAs coding for tumorigenesis and is tightly regulated at a translational level[198]. In addition to the length of the UTRs, the UTR associated mRNA elements also contributes to translational regulation. For instance, eIF4E mediated translation initiation of the 5'UTR of some mRNAs were preferred compared to the dependence on eIF4A[198]. The structural feature of some 5'UTRs associates with RNA binding proteins to regulate translation[198]. For example, during EMT, heterogenous ribonucleoprotein (HnRNP) E1 binds to TGFβ-activated translation, BAT element located on 3'UTR of specific mRNA transcripts important for EMT[199,200]. This results in the inhibition of

eEF1A1 dissociation from the A-site and thereby stopping elongation[199,200]. Elongation of these EMT regulating transcripts takes place upon TGFβ induced phosphorylation of HnRNP E1 which dissociates the BAT element[199,200].

Translation is also dysregulated in cancers at the level of elongation. Phosphorylation of elongation factor eEF2 by eEF2K plays a critical role in translation elongation regulation and is directly regulated by mTOR signaling[201,202]. eEF2K is downregulated in many cancers leading to continuous translation elongation of transcripts in tumor cells by induction of eEF2 activation[201,202].

1.5.5 Specialized ribosomes

Over the years, the conventional understanding of ribosomes as a static apparatus with only constitutive ability and non-regulatory functions has now been challenged by studies demonstrating ribosome heterogeneity[203,204]. Given that there are 80 ribosomal proteins, hundreds of ribosomes associated factors and several rRNA sequences, the ribosomes can be considered highly complex structures and almost per definition heterogenous[203,204]. Recent studies have showed that ribosomes can have specialized functions arising from variations in ribosomal proteins, post-translational modifications of ribosomal proteins and sequences of rRNA that make up the ribosomes[203–207]. More specialized translation by ribosomes can be attributed to its association to cis-acting elements with a specific mRNA subset. Specific ribosome associated factors can also lead to specific translation[208]. Examples of such factors are scaffold protein, protein kinase C (PKC) which associates with 40S subunit and its receptor RACK1 that interacts with various signaling molecules to make changes in the translation machinery[208]. Tissue specific allelic variants of rDNA genes in human and mice have also been found, though the functions of these variations remain unknown and to be functionally tested[205]. Heterogenous population of ribosomes in a single cell and its regulatory functions with regards to gene expression regulation and cell physiology are currently being discussed[203,204].

1.6 THERAPEUTIC POTENTIAL OF TARGETING RIBOSOME BIOGENESIS

Given that increased ribosome biogenesis and protein synthesis have been implicated in cancer, targeting ribosomal biogenesis and the translation machinery represents an attractive therapeutic target. This notion is further underscored by recent evidences of genetic mutations in ribosomal proteins and the idea of ‘specialized ribosomes’[204]. Disruption of ribosome biogenesis by inhibiting Pol I mediated rDNA transcription can be achieved with various

compounds including actinomycin D and BMH-21[209] (**Table 1**). Actinomycin D is a derivative of acridine, that intercalates into GC-rich regions of the rDNA genes and inhibits synthesis of rRNA by Pol I[209]. The rDNA promoter sequences are rich in G-quadraplex structures and compounds like CX-3543 and CX-5461 inhibits Pol I mediated rDNA transcription by binding to these quadraplex structures in proliferating cancer cells and prevents the association of Nucleolin (CX-3543) or SL-1 (CX-5461) to the promoter[111]. CX-5461 has been shown to have potent anti-tumor effect both *in vitro* and *in vivo* and is currently in clinical trial trials for hematological and breast cancer, making it the first Pol I inhibitor to enter into clinical trial[111]. This anti-cancer effect is thought to be mediated through both p53 dependent and p53 independent mechanisms. It has further been suggested that CX-5461 hampers the DNA replication fork by stabilizing the G4 DNA, which has led to its application in BRCA1 and BRCA2 deficient tumors where homologous recombination is altered[111]. Furthermore, platinum drugs like phenanthriplatin and oxaliplatin have shown to inhibit rDNA transcription as their main mechanism of action rather than only inducing conventional DNA damaging activity[209].

Tumors with increased ribosome biogenesis might also be able to evade immune responses by upregulated expression of programmed death ligands 1/2 (PD-L1 and PD-L2)[209]. Signaling transduction pathways like PI3K-AKT-mTORC and RAS/ERK that regulate ribosome biogenesis are dysregulated in many tumors and can be targeted by ribosome biogenesis inhibitors for effective therapy. Combination therapy using Pol I inhibitor CX-5461 and inhibitors against PI3K-AKT-mTORC1 dependent ribosome biogenesis showed additive effects in Eμ-MYC lymphoma mice suggesting its potential, for example in MYC driven cancers[210]. Collectively, these data suggest that ribosome biogenesis is an important target in cancer and further understanding of ribosome biogenesis and the translation landscape in cancer may open up new avenues for treatment of aggressive cancers that currently lack effective therapy.

Table 1. Ribosome biogenesis inhibitors

Drug	Target	Mode of action	Reference
Actinomycin D	GC-rich duplex DNA	Inhibits RNA Pol I-dependent transcription, promotes nucleolar disintegration	(Scala et al., 2016)
Avrainvillamide	NPM1	Promotes NPM1 dissociation in nucleolus	(Mukherjee et al., 2015)
Camptothecin	Topoisomerase I	Inhibits early rRNA processing, promotes nucleolar disintegration	(Burger et al., 2010)
Doxorubicin	Topoisomerase II, forms DNA intercalation	Inhibits RNA Pol I-dependent transcription	(Burger et al., 2010)
Flavopiridol	CDKs	Inhibits early rRNA processing, promotes nucleolar disintegration, downregulates RP expression	(Erol et al., 2017), (Burger et al., 2010)
Oxiliplatin	Forms DNA cross-links, transcription-translation mechanisms	Inhibits late rRNA processing	(Bruno et al., 2017)
CX-3543	rDNA G-quadruplexes	Inhibits RNA Pol I-dependent transcription elongation, promotes nucleolin redistribution	(Drygin et al., 2009)
CX-5461	rDNA G-quadruplexes	Inhibits RNA Pol I-dependent transcription initiation, promotes nucleolar disintegration	(Bywater et al., 2012)
5-Fluoroacil	Thymidylate synthase, incorporation into 47S pre-rRNA	Inhibits late rRNA processing	(Burger et al., 2010)
MG-132	Proteasome	Inhibits late rRNA processing	(Burger et al., 2010)
Mitomycin C	5'-CpG-3' guanosine alkylation in DNA	Inhibits RNA Pol I-dependent transcription, promotes nucleolar disintegration	(Snodgrass, Collier, Coon, & Pritsos, 2010)
Everolimus	mTOR	Inhibits rDNA transcription initiation	(Devlin et al., 2016)
BMH-21	rDNA GC-rich sequences	Inhibits RNA Pol I assembly	(Peltonen et al., 2014)

2 AIMS OF THE THESIS

The main objective of the thesis was to study role of ribosome biogenesis during tumor growth, progression and metastasis. Specifically, we investigated the dynamics and epigenetic control of *de novo* rRNA biogenesis and translation control underpinning the EMT program.

Specific aims of the thesis were the following:

1. Determine the role and epigenetic control of *de novo* rRNA biogenesis during EMT
2. Determine the role of Snail1in ribosome biogenesis during EMT
3. Determine and uncover translation control during EMT as a mechanistic link to *de novo* rRNA induced EMT
4. Determine the role of Wnt5a signaling in ribosome biogenesis in breast cancer

3 RESULTS

3.1 PAPER I

Ribosome biogenesis during cell cycle arrest fuels EMT in development and disease

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Induced rRNA synthesis is observed during EMT *in vitro* and *in vivo*

The mouse mammary epithelial cell line, NMuMG, which undergoes EMT after 48h of TGFβ treatment was employed to study rRNA synthesis during EMT. EMT was confirmed by decreased expression of epithelial markers E-cadherin (cdh1) as detected by immunofluorescence. Loss of coxsackie and adenovirus receptor (Cxadr or CAR) was also observed. Increased expression of mesenchymal proteins like N-cadherin (cdh2), Vimentin was observed in addition to increased stress fibre formation as detected by phalloidin stain. There was increased nuclear expression of EMT transcription factors Snail1, Smad4, and Twist. Reduced incorporation of EdU (5-ethynyl-2′-deoxyuridine) and Ki67 intensity confirmed the cell cycle arrested, non-proliferative state of NMuMG cells after EMT induction. NMuMG cells after 48h of TGFβ treatment showed increased rRNA synthesis assayed by 5-Fluorouridine (FUrd) incorporation. FUrd is incorporated into the nascent RNA. This increased rRNA synthesis was decoupled from proliferation and was accompanied by a modest reduction in global protein synthesis.

Similar pattern of increased rRNA synthesis decoupled from proliferation was observed in Py2T mammary cell line. This cell line is obtained from the MMTV-PyMT mouse and undergoes EMT upon 48h TGFβ treatment. Increased rRNA synthesis was also observed in MCF7 human breast cancer cell line that undergoes EMT after 48h under hypoxic conditions, confirming that the observed rRNA synthesis is not unique to the TGFβ stimuli nor cell type specific. The increased synthesis of rRNA during EMT was confirmed *in vivo* in delaminating neural crest cells of both chick and mouse embryos during development. The embryos pulsed with FUrd/EU and EdU/BrdU showed increased rRNA synthesis in the migrating cell populations whereas the cells that were proliferating did not overlap with the migrating cells as identified with Snail2 and Sox10. These models show that the observed induction in rRNA synthesis during EMT is conserved across species and various EMT-inducing stimuli and during development.

Increased ribosome biogenesis is observed during EMT and occurs during cell cycle arrest

We further confrimed increased rDNA transcription as measured by 45S pre-rRNA transcript after 48h of TGFβ treatment by qRT-PCR. Induced expression of processed rRNA transcripts (34S, 28S,18S, and 5.8S) were also observed. Pol I complex proteins including Pol I itself, UBF, RRN3 and rRNA processing proteins like, Nucleolin, Fibrillarin, Sirt7 and B23 were also upregulated after 48h of TGFβ treatment to accommodate the increased ribosome biogenesis program during EMT. To confirm the cell cycle stage accompanying this increased ribosome biogenesis, we employed the FUCCI technology. We observed cell cylce arrest and synchronization of cells at G1/S after 48h of TGFβ treatment which was accompanied by a decrease in cyclin D1 levels and increase in cyclin E levels. These observations show that there increased ribosome biogenesis can be detected during G1/S phase in EMT when the cells are cell cycle arrested.

Activation of rDNA Operons observed during EMT

We performed Chromatin immunoprecipitation assay (ChIP) analysis to study the changes in rDNA heterochromatin during the increased ribosome biogenesis that accompanies EMT. TIP5, a major component of the nucleolar remodeling complex (NoRC) repressive complex showed reduced association to the rDNA promoter and Snail1 promoter after EMT whereas an increased association was observed at the E-cadherin promoter. Increased recruiment of the Pol I complex components Pol I, UBF and Sirt7 to rDNA casette after EMT were also observed consistent with the observation of increased rRNA synthesis. These observations show an activation of rDNA operons taking place during EMT, that are otherwise silenced in the epithelial cells.

Regulation of rRNA synthesis during EMT by Snail1

Chip analysis revealed the recruitment of Snail1 to rDNA promoter, 18S and 28S regions post-EMT. To further confirm the role of Snail1 in regulation of rRNA synthesis during EMT we employed NMUMG-Snail1-ERT2 cells that undergo Snail1 driven EMT upon addition of 4-hydroxytamoxifen. We observed increased rRNA synthesis pulsing cells with EU (5-Ethnyl uridine) which similairly to FUrd gets incorporated into nascent rRNA trancripts, increased nucleolar UBF and Fibrillarin in this Snail1 driven EMT model. This finding shows that Snail1 directly regulates rRNA synthesis during EMT.

EMT is hampered by inhibition of ribosome biogenesis

To determine a potential driving role for *de novo* ribosome biogenesis in EMT, we inhibited ribosome biogenesis by disrupting Pol I complex assembly at rDNA promoters using a small molecule inhibitor of CX-5461, at a time point where the EMT process had been initiatied, i.e. 27h post TGFβ treatment. CX-5461 treatment reduced FUrd incorporation and 45S transcript levels concomitantly with a reduction in the association of UBF and Snail1 to the rDNA promoter. The invasive capacity of the TGFβ treated cells were significantly reduced upon treatment with CX-5461. We also observed a reduction in the mesenchymal marker Vimentin and stress fibre formation and Snail1 upon CX-5461 treatment. In addition to pharmacological inhibition, genetic silencing of the largest subunit of Pol I (Pol1ra) also lead to decreased invasiveness and reduced expressed of mesenchymal marker vimentin. These results indicate that *de novo* rRNA synthesis is essential to execute EMT.

Ribosome biogenesis during EMT is associated with mTORC2 signaling

mTORC2 is a key driver of EMT and its activation has been linked to its association with the mature ribosome. Rictor, a major component of the mTORC2 complex was found to be localized to nucleolus and expression was increased upon TGFβ induced EMT. RNase treatment resulted in the loss of nucleolar localization of Rictor in the cell, suggesting its association with RNA. CX-5461 treament reduced the expression of Rictor consistent with reduced mTORC2 signaling and diminished EMT program while mRNA levels of Rictor remained unchanged indicating that the response to CX-5461 is post-transcriptional.

Induced expression levels of Pol I and Rictor *in vivo* during tumor progression

We employed MMTV-PyMT mouse model to study the importance of rRNA synthesis during tumor progression. The MMTV-PyMT model mimics human breast luminal tumor development starting with hyperplasia and progresses through adenoma to early and late carcinoma. This mouse models starts to develop tumors at 6 weeks, shows evidence of micro-metastasis in the lungs at 8 weeks and progresses to carcinoma at 12 weeks. The 12-week tumors show increased expression of Pol I as shown by immunofluorescence, especially at the invasive front. Ki67 levels reduced as the tumor progressed suggesting that the observed increase in rRNA synthesis during tumor progression were coinciding with the non-proliferative cell population. Increased levels of Rictor were observed during tumor progression by IHC. Pol I and Ki67 staining detected in the primary tumor and lung metastases of the E0771 mouse model, that is a basal-medullary adenocarcinoma model, followed the same pattern.

Inhibition of ribosome biogenesis results in differentiation of primary tumors and reduces metastasis

To confirm the contribution of rRNA synthesis in cancer progression and metastasis, we treated the MMTV-PyMT mice with two doses of CX-5461 (50mg/kg and 87mg/kg). Both doses of CX-5461 reduced the primary tumor volume and significantly reduced metastasis. Histological evaluation of the CX-5461 treated tumors showed significant changes in the morphology where the tumor appeared to be regressed and differentiated. This differentiation was confirmed by increased expression of CK8/18 and nuclear ERα expression by IHC. The expression of Snail1/2 was also reduced in the tumors after CX-5461 treatment. These results show that treatment with CX-5461 halts the *de novo* rRNA synthesis program and therby reducing the cell's metastasizing capacity and as well as differentiating the tumors into a beningn phenotype.

Clinical relevance of the findings were verified in human normal breast and breast cancer tisuues. Pol I expression was induced in the tumor tissues as compared to the normal tissue. The expression of Pol I was especially high in the triple negative breast cancer compared to the ERα+ tumors. Though further studies are required, relevance of Pol I expression in human tumors suggests that Pol I can be a possible therapeutic target to inhibit the EMT associated ribosome biogenesis.

3.2 PAPER II

Ribosome biogenesis during the Epithelial-to-Mesenchymal Transition mediates a unique translation program

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EMT is accompanied by extensive changes in transcription and translation

To determine the translational control of EMT program we performed ribosome profiling on NMuMG post 48h of TGFβ treatment. Poly-A-selected mRNA sequencing was performed and combined with the ribosomal profiling data to elucidate the transcriptional and translational control of EMT. Sets of genes were discovered to be changed at the mRNA level but not at the level of ribosome protected fragments (RPF) is defined as 'translationally' controlled. Ribosome profiling demonstrated that 1336 genes (about 40%) of the 3,143 differentially expressed genes were found to be translationally controlled. These results show the existence of pervasive translational control during EMT which would have been missed by conventional mRNA sequencing studies. New genes identified by these profiling efforts were verified in dinstinct EMT models and in patient tissues.

Translational control of EMT is affected by ribosome biogenesis inhibition

To determine the relationship between active ribosome biogenesis and translational control of EMT, we treated the NMuMG cells with Pol I assembly inhibitor CX-5461 27h after TGFβ treatment. Ribosome profiling showed 1478 genes to be differentially expressed between TGFβ and TGFβ with CX-5461 while no changes were detectected at the level of mRNA. Ribosome concentration was found to be unchanged as revealed by polysome profiles. Out of the 3048 genes, 824 genes that were differentially expressed after TGFβ treatment were significantly changed after inhibition of ribosome biogenesis. About 712 genes out of 824 (85%) showed reverted profile matching to those of untreated cells suggesting that around 24% (712/3048) of the gene expression changes accompanying the EMT program is driven by active ribosome biogenesis. 50% (364/712) of this gene set was found to be translationally controlled and CX-5461 blocked the upregulation and downregulation of 185 and 179 translationally controlled genes respectively. These experiments highlight the existence of an EMT associated ribosome biogenesis program contributing to translational control during the EMT program.

mTORC1 signaling cascade is changed during ribosome biogenesis

Our profiling studies revealed that post TGFβ treatment mRNA transcripts encompassing short and less structured UTRs were reduced which have previously been suggested to be features

of mRNA transcripts that are regulated by mTOR signaling. Accordingly, this is suggesting that a downregulation of pro-proliferative mTORC1 signaling program occurs during EMT. Consistent with this, phosphorylation at serine 2448 of mTOR (p-mTOR 2448) and phosphosphorylation of elongation factor eEF2 was observed to be downregulated post EMT. Interestingly, these mTORC1 marks were found to be upregulated by CX-5461 treatment. These findings reveal that the reduction in rDNA transcription mediated by CX-5461 treatment specifically impacts the translational control of gene expression which is intimately linked to the pro-proliferative mTORC1 program.

Elongation Factors are altered during EMT

We further investigated the other elongation factors contained within the heavy complex given that eEF2 phosphorylation is a downstream target of mTORC1 complex. We observed changes in expression and localization of the other elongation factors and this correlated with that cells post TGFβ treatment were more sensitive when treated with the translation elongation inhibitor Didemin B. These data support the notion that there might be changes in the physical translation elongation machinery during the EMT program. Collectively, our results demonstrate that EMT is accompanied by pervasive translation control and identifies the translation machinery and ribosome biogenesis as critical features of EMT that can be selectively targeted for therapeutic intervention.

3.3 PAPER III

Wnt5a Signals through DVL1 to Repress Ribosomal DNA Transcription by RNA Polymerase I

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rDNA transcription is repressed by Wnt5a signaling and is mediated through DVL1

Non-canonical, Wnt5a has been shown to acts as tumor suppressor in breast cancer. rDNA transcription has been implicated in cellular proliferation and is known to be induced by oncogenic signals. To determine whether a soluble factor such as Wnt5a could exert its tumor suppressive effect through regulation of rRNA biogenesis we determined the 47S pre-rRNA transcript in MCF7 cells after treatment with Wnt5a and found that the 47S pre-rRNA transcript to be reduced by more than 50%. This reduction was abrogated by a Wnt5a antagonist. Pre-nascent rRNA synthesis as denoted by FUrd incorporation was also reduced by 60%. This repression of rDNA was found to be mediated by gain of DVL1 and a loss of SIRT7 as revealed by the ChIP assay to the rDNA promoter, 18S and 28S regions in Wnt5a-expressing MCF7 cells. DVL1 was expressed in the nuclear and sub-nuclear regions co-localizing with Fibrillarin, whereas DVL2 and DVL3 were distributed in the cytoplasmic region, as detected by immunofluorescence. Stable transfection of MCF7 and BT549 cell lines with shRNAs targeting DVL1 resulted in increased 47S pre-rRNA transcripts accompanied by enlarged nucleoli as visualized by AgNOR. Treatment of the shRNA control cells with Wnt5a reduced 47S transcripts whereas the cells with DVL1 shRNA did not show any significant decrease in rDNA transcription. The results observed shows that Wnt5a signaling acts as a tumor suppressor by repressing rDNA and this suppression is mediated by gain of DVL1 and a loss of SIRT7, suggesting a role of DVL1 as a repressor of Pol I mediated rDNA transcription.

rDNA is regulated *in vivo* by Wnt5a signaling

To determine the effect of Wnt5a signaling on rRNA biogenesis *in vivo*, we employed MMTV-PyMT/Wnt5a^{++} mice. The Wnt5a null mice showed increased expression of Ki67, Sirt7 and enlarged nucleolar regions in their tumors when compared to the Wnt5a^{++} mice supporting a tumor suppressive role of Wnt5s by decreasing rDNA transcription resulting in halted tumor growth. Survival curves from the Cancer Genome Atlas (TCGA) showed poor prognosis to be associated with low expression of Wnt5a, SIRT7 and DVL1. These data support the role of Wnt5a signaling to suppress tumor development *in vivo* by regulating rDNA transcription.

4 DISCUSSION AND CONCLUSION

Our studies have revealed that the EMT program is accompanied by an induction of rRNA synthesis that occurs in the absence of cellular proliferation which is independent of stimuli or species. We have also shown that blocking *de novo* rRNA synthesis using Pol I assembly inhibitors hampers EMT and subsequent metastasis. These findings challenge the previous dogma, that ribosome biogenesis is only necessary for dividing cells and that it occurs without phenotypic specificity.

Interestingly, the EMT cell fate switch driven by *de novo* ribosome biogenesis occurs at G1/S phase of the cell cycle where they are in a cell cycle arrested state. These results also suggest that rRNA biogenesis regulation during cell cycle may be distinct in proliferative cells compared to cells undergoing EMT. Correspondingly, our investigations suggest that Pol I activities much like has been observed for Pol II are also regulated during the cell cycle, and in particular during cell cycle arrest at the G1/S transition[211].

The observed *de novo* ribosome biogenesis was due to the transcriptional activation of the rDNA genes that are otherwise silenced by TIP5, a component of the NORC repressive complex which dissociates from rDNA genes during EMT. That silenced rDNA genes are activated during EMT is in line with that EMT is a developmental program which is reactivated in cancer. This suggest the possibility of formation and activation of specific transcriptional complexes that interact with Pol I which may be necessary to transcribe a specific set of EMT associated rRNAs genes that will ultimately make up the mature ribosome. Animal cells contain approximately 300 copies of the mammalian rDNA genes as highly homologous and repetitive rDNA repeats located at chromosome 13, 14, 15, 21, and 22[212,213]; in mice, and in humans 12, 15, 16, 18 and 19[214–216]. Remarkably, only one *precise* rDNA sequence of the hundreds found in the mammalian genome are currently known. It therefore possible that different transcriptional complexes with factors such as Snail are activating unique rDNA sequences at distinct chromosomes resulting in the expression of distinct ribosomes. Recent studies supports this notion as it has been shown that in bacteria nutrient limitation-induced stress changes the expression of encoded rDNA genes which alter the rRNA composition within the assembled ribosome[217]. The expression of these conserved rRNA sequence variations within the assembled ribosome resulted in gene expression changes which results in changes in cell physiology[217]. Future studies are now needed to test if these encoded rRNA variants in a similar manner play a regulatory role in mammalian cells and specifically during EMT.

Our observation of the recruitment of Snail1 to rDNA promoter during EMT led to our discovery of Snail1 driving rRNA synthesis, which further suggests that Snail1 might directly or indirectly associated with the Pol I complex regulating 'EMT associated' rDNA transcription. Our findings extend Snail's previous extensive role in Pol II regulation during EMT to the Pol I machinery potentially functioning as a pan-RNA polymerase regulator much like the oncogene Myc. It also supports a role for Snail not only as a transcriptional repressor but transcriptional activator. Further studies will be needed to determine whether Snail also regulates Pol III transcription. Moreover, it will be interesting to determine which other components in addition to Snail1 that is driving the EMT- associated biogenesis program.

While the localization of Rictor, the defining component of the mTORC2 complex, in proliferating cells was primarily cytoplasmic, we observed increased nucleolar signal of Rictor during EMT. This rRNA dependent nucleolar localization of Rictor suggests a novel role of newly synthesized rRNA in modulating the mTORC2 kinase activity. This opens up the possibility of that newly synthesized rRNAs display a preference for specific associated factors, including Rictor, that can bind to the ribosomes and modulate the signaling cascade leading to the cell identity switch. There could also be other proteins interacting with these newly synthesized rRNAs contributing to a 'ribo-interactome' that modulates specialized translation based on cues from the interactome profile. Further experiments are required to study this potential regulatory axis, for example by further defining the interactome of ribosomes in proliferating and EMT cells. The association of Rictor to the newly synthesized rRNAs therefore supports a dual role of ribosomes: that they function as both regulators of translation and "signaling centers" that execute specialized cellular signaling cascades and functions. This preference for associated proteins may stem either from the encoded cues of the transcription complex or the sequence variation of rRNAs that are specified to carry out distinct cellular programs as mentioned above. Given that this model is correct, it is not absolute number of ribosomes being generated during EMT rather which ribosomes that are generated given that the number of ribosomes in a cell far exceeds the amount of Rictor molecules.

The observed induction in rRNA synthesis during EMT was not linked to an increase in global protein synthesis, despite the increase in mesenchymal proteins including Snail, Vimentin and N-cadherin. Together, these data could potentially suggest a role of the newly synthesized rRNA in forming ribosomes that translate mRNAs associated with the mesenchymal phenotype. Another interpretation is that the newly synthesized rRNA transcripts might act by sequestering factors responsible for pro-proliferative translation, thereby favoring translation

of mesenchymal proteins. All these findings suggest a plethora of other ways by which these newly synthesized rRNAs could be functioning to drive EMT in cancer progression.

In line with our *in vitro* findings, increased Pol I expression was observed during cancer progression in a metastatic mouse model and in human triple negative breast tumor samples. These expression data support the clinical relevance of our findings and the importance of targeting ribosome biogenesis. Mice treated with CX-5461 exhibited significantly reduced primary and secondary tumors. Importantly, the primary mouse tumors were differentiated into ERα positive phenotype with a more benign morphology after inhibition of ribosome biogenesis. This opens up a new therapeutic possibility of targeting aggressive TNBC and potentially render these tumors responsive to hormonal therapy. Another possibility to explain our findings could be that the Pol I inhibitor might specifically targeting the mesenchymal invasive cell population with the induced *de novo* ribosome biogenesis program resulting in a smaller epithelial tumor that is benign and with more proliferative properties which potentially can be targeted with conventional anti-proliferative therapies.

To gain mechanistic insights into the role of ribosome biogenesis during EMT a modified ribosomal profiling method combined with RNA sequencing efforts revealed that there were pervasive changes both at transcriptional and translation level during EMT. A significant percentage of the translationally controlled genes changed when ribosome biogenesis initiation was inhibited whereas there were no significant changes in the transcriptionally controlled genes. These data suggest that the newly synthesized rRNA driving the EMT program might have a regulatory role in controlling the translation of specific transcripts that are responsible for the loss of an epithelial cell phenotype and for the acquisition of mesenchymal phenotype. These studies also support the notion that the newly generated ribosomes during EMT may be distinct in their composition and participating in cellular signaling due to their interactome. Future studies will be necessary to determine how such ribosomes are assembled, what specific factors these ribosomes are binding, the stability of such ribosomes, how these ribosomes are capable of activating signaling cascades and how these ribosomes ultimately are linked to cell identity and metastasis. Such studies may reveal important insights into novel means of therapeutically targeting metastatic disease by identifying physical distinctions in the translation machinery and the unique features associated with the EMT-specific gene expression program.

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